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Nearest neighbor analysis of D1 and D2 subunits in the photosystem II reaction center using a bifunctional cross-linker, hexamethylene diisocyanate

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Abstract

A cross-linked product between the D1 and D2 subunits was generated by treating isolated spinach PS II reaction center with a bifunctional cross-linker, 1,6-hexamethylene diisocyanate. To obtain information on the contact site(s) between D1 and D2 proteins, the cross-linked product was cleaved by CNBr, the resultant fragments were separated using a reverse-phase HPLC, and then the partial sequence of the cross-linked polypeptide fragments were determined. By comparing the results with the deduced amino acid sequence of the D1 and D2 proteins from spinach, it is concluded that the C-terminal domains of the D1 subunit (D308–A334) and that of the D2 subunit (Y297–L353) are in close proximity.

Key words: Chemical cross-linking; D1 protein; D2 protein; Hexamethylene diisocyanate; Photosystem II; Reaction center

1. Introduction

The isolated photosystem II (PS II) reaction center consists of five protein subunits, i.e. the D1 and D2 proteins, α- and β-subunits of cytochrome b_{559} and *psb*I gene product [1,2]. Among these subunits, both the D1 and D2 proteins are important in the function of PS II in that they provide the binding sites for pigments and redox co-factors, such as chlorophyll *a*, pheophytin *a* and β-carotene, and furnish machinery for the primary charge separation [3]. The D1 and D2 proteins are significantly homologous in the amino acid sequence to the L and M subunits of purple bacterial reaction center, the structure of which has recently been determined by X-ray crystallographic analysis [4–6]. In addition to the sequence homology in the constituent subunits mentioned above, the PS II and purple bacterial photosystem have been shown to be similar in their functional organization, especially on the reducing side, as demonstrated by kinetic, optical and EPR analyses [7]. Thus it is generally assumed that the PS II reaction center has a similar structural organization to that of its purple bacterial counterpart. However, the PS II reaction center has quite a unique function to generate high oxidizing power which eventually cleaves water to evolve oxygen. Thus it is natural to expect some basic differences between both photosystems in the molecular organization of proteins, especially on the donor side.

At present, in order to understand the gross protein structure of the PS II reaction center, chemical cross-linking analysis is a useful method, as shown by a previous study [8], since no preparation of PS II suitable for crystallographic analysis is so far available. In the present study, the chemical cross-linking analysis was focused on determining the contact site(s) between the D1 and D2 proteins in the PS II reaction center, in order to provide basic information for the forthcoming structural analysis using crystals. For this purpose, 1,6-hexamethylene diisocyanate (HMDI) was employed with the isolated PSII reaction center complex prepared from spinach. The major conclusion from this analysis is that the carboxyl-terminal domains of both the D1 and D2 proteins are in close proximity, at least at a specific site(s) in the PS II reaction center.

2. Materials and methods

The PS II reaction center complex was prepared from spinach grana thylakoids as described by Nanba and Satoh [1], with a modification; Triton X-100 in the buffer solution was exchanged with digitonin (0.2%) by a column procedure.

For cross-linking, HMDI was added to the isolated PS II reaction center complex (0.4 mg·chl·ml^{-1}) dissolved in a 40 mM MES-NaOH (pH, 6.5) buffer containing 0.2% digitonin. The cross-linker was freshly prepared by dissolving in dimethylsulfoxide prior to each experiment and the concentration of reagent in the reaction mixture was kept at less than 4%. The cross-linking reaction was carried out at room temperature for 10 min and then terminated by adding glycine in 100-fold molar excess over the cross-linker. For analyzing cross-linked products, the sample was treated with 3% lithium dodecyl sulfate and 60 mM dithiothreitol for 30 min at room temperature, and then separated by SDS-PAGE by the method of Laemmli [9], with modifications described in [10]; analyzing gels contained 6–22% acrylamide and 7.5 M urea. After electrophoresis, the separated proteins in gels were transferred onto polyvinylidine difluoride (PVDF) membranes and detected by Ponceau S staining. Each stained band was cut out and then destained in 0.2 N NaOH solution. To elute proteins, the PVDF membranes were incubated in a solution containing 20% acetonitrile and

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70% formic acid for 2 h and then proteins in the eluent were concentrated by centrifugation. The cyanogen bromide (CNBr) cleavage of proteins in solution was conducted at 10 mg·ml^{-1} CNBr in 70% formic acid for 72 h at 25°C. After CNBr cleavage, peptide fragments were lyophilized and then dissolved in a solution containing 0.1% trifluoroacetic acid. The peptide fragments were separated by reverse-phase HPLC with a Cosmosil 8 ODS column (2.1 × 150 mm; pore size 5 μm) using a 5–90% acetonitrile concentration gradient containing 0.1% trifluoroacetic acid at a flow rate of 0.3 ml·min^{-1} and processed for amino-terminal sequence analysis. The amino-terminal sequence of each component was analyzed by an Applied Biosystem Model 477A sequencer. For immunoblotting, proteins resolved by SDS-PAGE were transferred to a nitrocellulose membrane and detected by an enzyme-linked immunoblot analysis [11]. The specific antibodies raised against each component of the PS II reaction center complex were provided by Dr. Ikeuchi (University of Tokyo).

The fluorescence spectra of PS II reaction centers were measured using a Hitachi 850 spectrofluorometer; the spectra were corrected for the sensitivity of the instrumental response [12]. For measuring the light-induced absorbance change at 545 nm, samples in a 10-mm cuvette were cross-illuminated in an Hitachi 356 spectrophotometer with an actinic light from a xenon lamp (500 W) at 25°C. Appropriate filter combinations were used for protecting the photomultiplier from strong actinic light as follows; a Corning 4-96 filter for measuring beam and a red cut-off filter (VR-67, Toshiba, Tokyo) for actinic beam. The sample, in a 30 mM MES-NaOH (pH 6.5) buffer, containing 1 μM methyl viologen and 3 mg of sodium dithionite per ml, was incubated for a few minutes before each measurement as shown in [1].

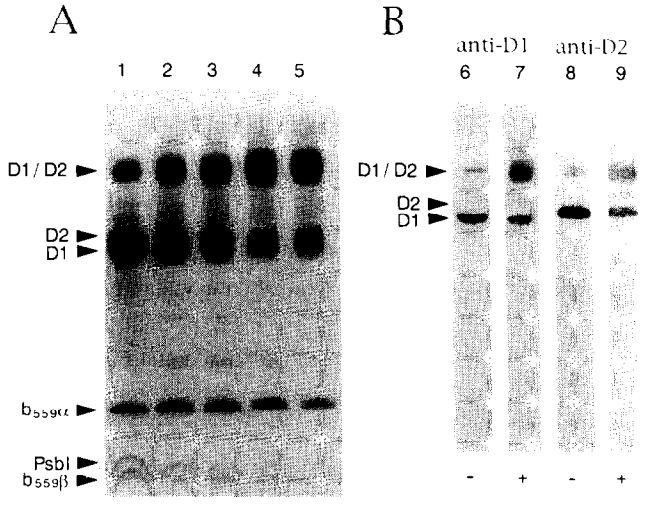

Fig. 1. HMDI treatment of the PS II reaction center complex. (A) SDS-PAGE profiles of HMDI-treated PS II reaction centers. The PS II reaction center complex was treated with 0 (lane 1), 0.0096% (lane 2), 0.013% (lane 3), 0.02% (lane 4) or 0.08% (lane 5) HMDI at 25°C for 10 min. (B) Immunoblot analysis of cross-linked products. PS II reaction center complex was incubated in the absence (lanes 6,8) or presence (lanes 7,9) of 0.038% HMDI. Polypeptides were detected by antibodies raised against D1 protein (lanes 6,7) and D2 protein (lanes 8,9), respectively.

3. Results and discussion

Fig. 1A shows the CBB-stained polypeptide profile analyzed by SDS-PAGE for the PS II reaction center complex treated with different concentrations of HMDI. Each polypeptide band, except for the β-subunit of cytochrome b_{559}, in the untreated sample (lane 1), was identified as indicated on the left side of the figure, using specific antibodies raised against the individual components. At pH 8.0, HMDI treatment of the PS II reaction center complex resulted in the formation of two cross-linked products, i.e. a 15 and a 65 kDa component, as described in [8]. In the previous analysis, the 15 kDa component was assigned to be a cross-linked product between the α-subunit of cytochrome b_{559} and *psb*I gene product; amino acid residues engaged in the cross-linking were identified as Lys-4 of the *psb*I gene product and the amino-terminal α-amino group in the α-subunit of cytochrome b_{559} [8]. The other component, which is more predominant from acidic to basic pH, has a size that almost exactly corresponds to that of the D1/D2 heterodimer. At the pH used in this experiment (pH 6.5), HMDI- treatment in the range of 0.01–0.08% resulted in the formation of the 65 kDa band in a concentration-dependent fashion (Fig. 1A), which was accompanied by a parallel decrease in the intensity of both the D1 and D2 bands at 32–34 kDa; at 0.038% HMDI, more than 90% of the monomeric bands disappeared, accompanied by a corresponding increase in the 65 kDa band. This strongly suggests that the 65 kDa band is a heterodimer formed between the D1 and D2 proteins, rather than the homodimers. The immunological analysis using antisera raised against each component confirmed that this component contains both D1 and D2 proteins, as shown in Fig. 1B.

In order to determine whether the PS II reaction center complex is still active in primary photochemistry and is not modified appreciably in structure even after thorough HMDI treatment to form D1–D2 heterodimers in most of the complexes (0.038% HMDI), both the fluorescence emission and the kinetics of photoaccumulation of the primary electron donor (pheophytin) in the reaction center were analyzed (data not shown). The fluorescence was excited at 435 nm (chlorophyll *a* band) at 77 K for both untreated and the HMDI-treated PS II reaction center complex. The spectrum for both samples, as well as the relative quantum yield of fluorescence, was practically identical, except for a minor change in the higher wavelength region, suggesting that the entire energy transfer system in the complex, which sensitively reflects protein structure around pigments, is not affected appreciably by the treatment. The intactness of the reaction center in terms of primary photochemistry was also demonstrated by the kinetic measurement of photoaccumulation of pheophytin anion under steady-state illumination at room temperature (not shown). Based on the results described above, it is reasonable to expect that the structure of proteins in the PS II reaction center is not modified appreciably even after the HMDI-treatment to form D1–D2 heterodimers.

In order to specify the site(s) of cross-linking between the D1 and D2 proteins, the cross-linked product (D1–D2 heterodimer), obtained by HMDI treatment at 0.038% at pH 6.5, was cleaved by CNBr as described in

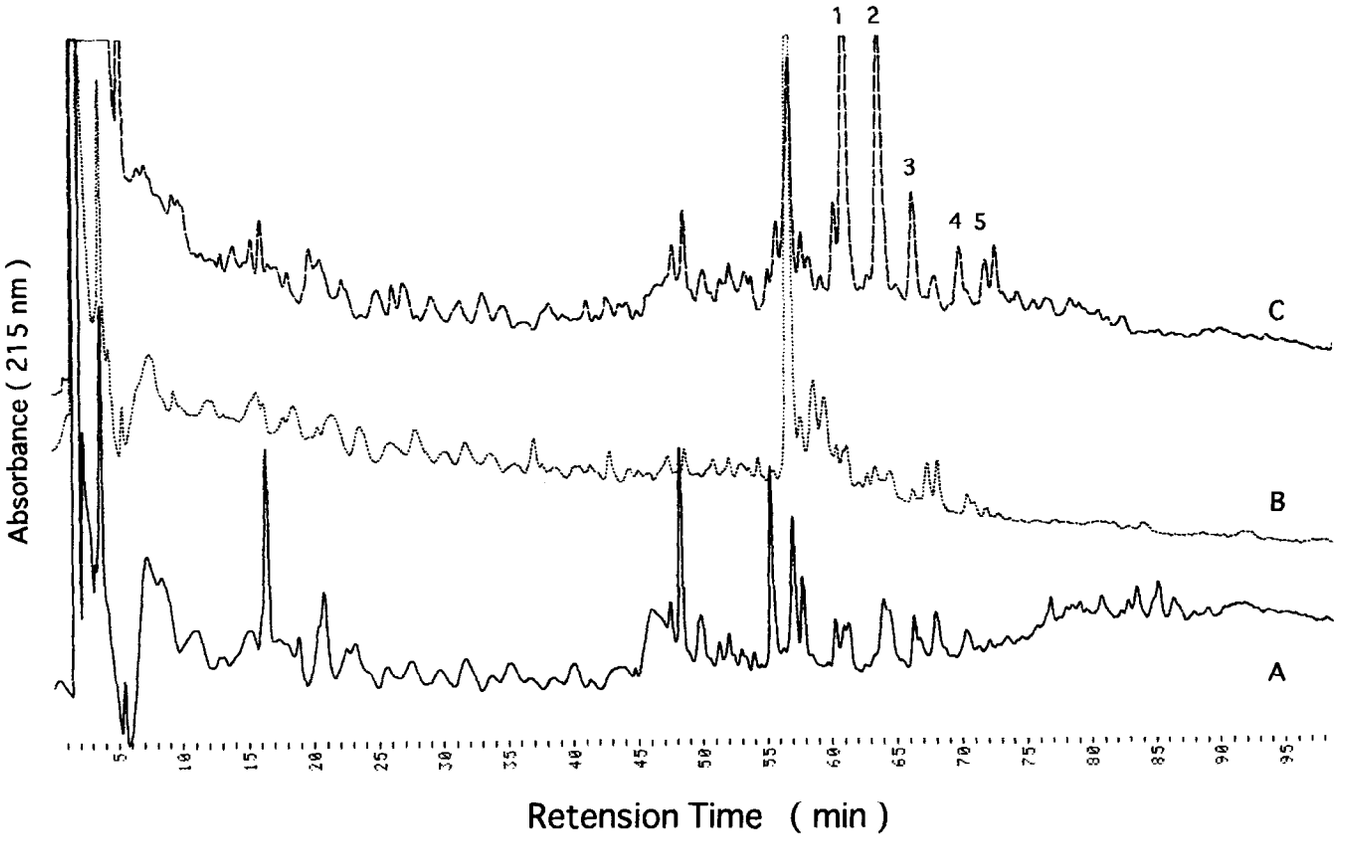

Fig. 2. Elution profiles of a reverse-phase HPLC of CNBr fragments obtained by HMDI treatment. (A) D1 protein; (B) D2 protein; (C) D1–D2 heterodimer. See text for further explanations.

section 2 and then the cross-linked products were analyzed by HPLC using a reverse-phase small bore column as shown in Fig. 2. The deduced sequence of the D1 and D2 proteins of spinach contain 11 and 8 cleavage sites, respectively, for CNBr, i.e. the carboxyl-side of methionine [13], and thus the HMDI treatment is expected to produce 12 and 9 fragments, for the respective protein, as compiled in Table 1.

Fig. 2 shows the profile of the CNBr fragments separated by HPLC after 72 h of cleavage at 25°C, for the D1 protein (Fig. 2A), the D2 protein (Fig. 2B), and the cross-linked product between the D1 and D2 proteins (65 kDa component) produced by HMDI treatment at 0.038% (Fig. 2C). From the figure, it is evident that there are at least five major bands (numered 1–5) for the D1–D2 heterodimer which are not present in the profile of the constituent components. The three major fractions (peaks 1, 2 and 4) were collected and then subjected to amino-terminal sequence analysis as described in section 2. Peak 1 gave no amino acids from the first to the tenth cycle of Edman degradation, probably because of amino-terminal blockage, modification and/or its low yield by unknown reasons. Exactly identical pairs of PTH derivatives were detected in most cycles of Edman degradation for both peaks 2 and 4, i.e. from the 3rd to the 7th cycle and 9th cycle (Table 2). By comparing the result obtained by this analysis with the deduced sequence for CNBr fragments compiled in Table 1, the two components, peaks 2 and 4, were concluded to be cross-linked products formed between the D1 and D2 proteins, i.e. the fragment of the D1 protein starts from the 294th position (A-F-N-L-N-G-F-N-F-N- - - -) and that of the D2 protein starts from the 276th position (A-F-V-P-V-T-

Table 1. Reduced amino acid sequence of CNBr fragments from spinach D1 and D2 proteins

Fragment No.		Amino Acid Sequence	No. of amino acids
D1 protein			
	1	TAILERRESESLWGRFCNWITSTENRLYIGWFGVLM	36
	2	IPTLLTATSVFIIAFIAAPP・・・・FLLGVACYM	90
	3	GREWELSFRLGM	12
	4	RPWIAVAYSAPVAAATAVFLIYPIGQGSFSDGM	33
	5	PLGISGTFNFM	11
	6	IVFQAEHNILM	11
	7	HPFHM	5
	8	LGVAGVFGGSLFSAM	15
	9	HGSLVTSSLIRETTENES・・・・IWFTALGISTM	79
	10	AFNLNGFNFNQSVVDSQGRVINTWADIINRANLGM	35
	11	EVM	3
	12	HERNAHNFPLDLA	13
D2 protein			
	1	TIAVGKFTKDEKDLFDSM	18
	2	DDWLRRDRFVFVGWSGLLL・・・・ALIGFM	107
	3	CQLGGLWAFVALHGAFLRQF・・・・WTLNPFHM	71
	4	M	1
	5	GVAGVLGAALLCAIHGATVENTLF・・・・AEETYSM	47
	6	VTANRFWSQIFGVAFSNKRWLHFFM	25
	7	LFVPVTGLWM	10
	8	SALGVVGLALNLRAYDFVSQEI・・・・LNEGIRAWM	48
	9	AAQDQPHENLIFPEEVLPRGNAL	23

The numbering of the amino acids starts from the amino-terminal methionine. Internal sequences are abbreviated by dots.

Table 2
Amino-terminal sequence analysis of CNBr fragments obtained by HMDI-treatment of PS II reaction center

Peptide fragment	Sequencing cycle									
	1	2	3	4	5	6	7	8	9	10
Peak 2	-	F	N	L	N	G	F	-	F	N
	-	F	V	P	V	T	G	L	W	-
Peak 4	-	F	N	L	N	G	F	N	F	N
	-	F	V	P	V	T	G	L	W	-

Bars indicate where unequivocal amino acid assignments could not be made. See text for further explanations.

G-L-W-M- - - -). The fact that we obtained essentially the same amino-terminal sequence profile for the two components means that the cleavage reaction was not complete under the conditions used in this experiment. This often happens, for example, due to structural hindrance in this kind of analysis. Thus the 10th, 11th and 12th fragments of the D1 subunit and the 7th, 8th and 9th fragments of the D2 subunit shown in Table 1 have to be taken into account for predicting amino acids involved in the cross-linking. On the other hand, HMDI has a chain length of 11 Å and is known to react with a wide variety of functional groups, such as amino, sulfhydryl, imidazole, aromatic hydroxyl and carboxyl groups, at pH 6.5 [15], and thus the fragments of the D1 and D2 proteins discussed above contain 7 and 15 reactive candidates, respectively. This makes it impossible to specify the amino acids directly engaged in the cross-linking in this analysis. However, we can restrict the sequence domain of the proteins, based on the deduced primary structure, to P308–A344 for the D1 protein and Y297–L353 for the D2 protein.

According to the two-dimensional model proposed by Trebst [14], the regions of the D1 and D2 proteins engaged in the cross-linking suggested in this study correspond to the long hydrophilic carboxyl-terminal extensions supposedly located in the lumenal space of the thylakoid. Thus it is predicted, based on the present analysis, that the carboxyl-terminal extension of both the D1 and D2 proteins have a cross-contact site(s) separated by less than 11 Å, and thus both domains are in close proximity in the PS II reaction center. This may be important in organizing the oxygen-evolving machinery in PS II since a recent analysis using site-directed mutagenesis has suggested that these parts of the proteins are important in the Mn or Ca binding [16,17]. In this context, it is interesting to compare the carboxyl- terminal regions of the D1 and D2 proteins with those of the L and M subunits of the purple bacteria reaction center. The L and M subunits of purple bacteria reaction centers have short carboxyl-terminal extensions which are present far apart from each other, as demonstrated by crystallographic analyses [4–6,18], whereas the putative structure of the D1 and D2 proteins has a long carboxyl-terminal extension of 50 and 61 amino acid residues, respectively. Moreover, there is no significant homology between the carboxyl-terminal extension of the D1–D2 proteins and that of the L–M subunits of purple bacteria. The extensions of the D1 and D2 proteins, on the other hand, are highly conserved among higher plants, algae and cyanobacteria. Therefore, it is natural to speculate that the cross-contact between the D1 and D2 proteins, with a distance of less than 11 Å, at least at specific side chains in the carboxyl-terminal extension, might be important for a specific function of PS II, possibly in organizing the water splitting machinery.

In conclusion this analysis clearly demonstrated that the carboxyl-terminal domains of both the D1 and D2 subunits are in close proximity in the PS II reaction center, and suggested that further analysis using other cross-linkers may provide important and specific information as to the gross structural organization of polypeptides in the PS II reaction center complex.

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